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PROTOCOL FOR MEASURING CIRCULATING LEVELS OF GONADAL SEX STEROIDS IN FISH

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ABSTRACT

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Alterations in circulating levels of sex steroids have been found in fish captured near bleached kraft pulp mills, and have been associated with a number of other physiological and whole organism responses. The Environmental Effects Monitoring Requirements for Pulp and Paper Mills recommends the measurement of gonadal sex steroids as a sensitive indication of the potential for reproductive impacts. Circulating levels can be measured with a variety of methods, but the only approach currently satisfying the requirements of being sensitive to picogram (pg) levels and being widely commercially available is radioimmunoassay (RIA). There is no universal protocol for steroid hormone analysis by RIA; this report outlines an assay protocol that has been utilized successfully for the past 12 years in a research laboratory, and for which, all reagents and equipment are easily available commercially.

RÉSUMÉ

McMaster, M.E., K.R. Munkittrick and G.J. Van Der Kraak. 1992. Protocol for measuring circulating levels of gonadal sex steroids in fish. Can. Tech. Rept. Fish. Aquat. Sci. 1836: 29 p.

Des modifications du taux de stéroïdes sexuels circulants ont été observées chez des poissons capturés à proximité des usines de pâte kraft blanchie et ces modifications ont été liées à d'autres effets physiologiques ainsi qu'à d'autres réactions de l'organisme entier. Les exigences en matière de surveillance des effets environnementaux pour les usines de pâtes et papiers recommandent la mesure des stéroïdes sexuels gonadiques comme une indication sensible d'effets potentiels sur la reproduction. Il existe plusieurs méthodes pour mesurer les taux de stéroïdes circulants, mais le radio-immunodosage (RIA) est la seule méthode qui répond aux exigences, à savior une sensibilité de l'ordre du picogramme et une grande accessibilité commerciale. Il n'y a pas de protocole universel pour l'analyse des hormones stéroïdes par RIA, mais dans le présent rapport, on décrit un protocol utilisé avec succès depuis 12 ans par un laboratoire de recherche; de plus, on peut se procurer facilement dans le commerce tous les réactifs et appareils nécessaires à la réalisation du protocole.

PREFACE

This document was prepared in response to requirements for protocols under Environmental Effects Monitoring (EEM) Programs proposed as part of revisions to Canada's Pulp and Paper Mill Effluent Regulations, which will be promulgated in early 1992. Research programs have consistently demonstrated altered steroid metabolism in fish exposed to bleached kraft pulp mill effluents. The alteration in steroid metabolism was associated with other impacts, including induced hepatic mixed function oxygenase enzymes, altered fecundity, increased age to maturity, decreased secondary sexual characteristics and altered growth in wild fish. This protocol is intended for use by biologists with laboratory experience in biochemistry and should be an integral part of EEM programs, as a step in tiered-testing to interpret the impacts of chemical contamination on fish reproductive development and performance.

1.0 INTRODUCTION

Monitoring the effects of industrial wastes in receiving waters includes monitoring the biological responses of fish. The induction of the hepatic mixed function oxygenase (MFO) system has been shown to be a good indicator of exposure to bleached kraft mill effluent (BKME) (Hodson *et al.* 1991). In studies which have measured gonadal sex steroids, levels have been altered when MFOs have been induced. These changes have been associated with alterations in gonadal size, fecundity, age to maturation and secondary sexual characteristics, and are believed to impact fish population-level responses.

This protocol document describes the measurement of circulating levels of gonadal sex steroids, as a tool for monitoring reproductive impacts of chemical contamination associated with bleached kraft mill effluent and induced hepatic MFO levels. It includes the measurement of two sex steroids in fish blood and it identifies what data must be collected, when and how it is to be collected, and how positive and negative responses are to be interpreted.

1.1 Rationale

In spite of the fact that many biochemical indicators and physiological processes are sensitive to BKME-mediated alterations, many of these responses are of limited use in assessing damage to aquatic ecosystems because they are not linked to effects at the population level. However, changes associated or correlated with deleterious effects on reproductive processes can be used to diagnose damage in aquatic populations. Even small decreases in reproductive success may be associated with population declines (Cushing 1979).

Field studies on seasonal variation of sex steroid levels have been completed on a

number of species and reductions in circulating levels of sex steroids reliably indicates exposure to compounds impacting the reproductive system. Recently, field studies have incorporated the measurement of sex steroid levels in attempt to correlate reproductive problems with exposure to PCBs (Spies *et al.* 1988a,b) and BKME (Munkittrick *et al.* 1991a 1992a,b; Hodson *et al.* 1992; McMaster *et al.* 1992). Scandinavian, and Canadian studies of fish captured near sources of BKME have shown dramatic impacts on reproduction when compared with fish from reference sites (Andersson *et al.* 1987 1988; Munkittrick *et al.* 1991a 1992b; Hodson *et al.* 1992; McMaster *et al.* 1992). These include delayed sexual maturation, reduced gonad growth, reduced fecundity with age, reduced egg size, and reduced secondary sexual characteristics. Although the number of species studied has been small (lake trout, lake whitefish, white sucker, longnose sucker, starry flounder and perch) the results are consistent qualitatively. Species differences in the magnitude of the responses are found, and both biological variables (sex, age, time of day, spawning status, size, genetic strain) and habitat variables (temperature) influence the response.

The measurement of hepatic MFO enzymes is required under the Aquatic Environmental Effects Monitoring (EEM) Requirements for pulp mill effluents (Environment Canada 1991a), as an indicator of exposure to pulp mill effluent. Although induced levels of enzymes have been consistently found at bleached kraft pulp mills, the increases have not been correlated with any specific chemicals, and the consequences of increased catalytic activity are currently unknown. The MFO system facilitates the excretion of many xenobiotics by enhancing their hydrophilicity through the addition of polar groups. In addition to this role in the elimination of lipophilic contaminants, MFOs are important in the synthesis and metabolism of endogenous compounds including steroids, fatty acids and prostaglandins (Hall 1985; Rattner *et al.* 1989). It has been hypothesized that increased MFO activity may be responsible for the impacts of many lipophilic contaminants on reproduction (Truscott *et al.* 1983; Okey 1990).

Hormones from the gonads (androgens, estrogens, and progestogens) and the anterior

pituitary (gonadotropin hormones) interact to regulate growth and the maintenance of reproductive organs, production of gametes, and the control of sexual behaviour and secondary sexual characteristics. Interruptions or changes in the control, the production, or metabolism of these hormones can result in delayed maturity, reduced gamete growth, and altered spawning behaviour. Studies which have examined steroids at an appropriate stage of gonadal development have demonstrated reduced levels of plasma sex steroids (testosterone, 11-ketotestosterone, 17β-estradiol and 17α20β-dihydroxy-4-pregnen-3-one) in fish exposed to bleached kraft pulp mill effluent relative to fish at reference sites (Munkittrick *et al.* 1991a 1992a,b; McMaster *et al.* 1992). These studies are consistent with results showing altered levels of steroids (Lindstrom-Seppa and Oikari 1989; Hodson *et al.* 1992) and have been correlated with changes in reproductive development and/or performance.

It should be emphasized that there may be as-of-yet unidentified impacts on fish populations which operate independently of MFO induction and steroid disruption. At this point in time, measurement of MFO activity represents one of the easiest and most sensitive responses of exposure to pulp mill effluent. The area in which MFO enzymes are induced is assumed to represent the zone of direct influence of the effluent on fish physiology and performance (Hodson *et al.* 1991), while the zone in which steroid levels are altered is assumed to represent the maximum extent of direct influence of the effluent on fish reproduction. It is currently unknown how steroidal control is altered by exposure to pulp mill effluent, and what relationship the steroidal problems have with MFO induction. It is unknown, at this time, if steroidal problems exist at pulp mills in the absence of MFO induction, although it is well documented that high levels of reproductive steroids associated with the prespawning period result in depressed levels of hepatic MFO activity (Munkittrick and Van Der Kraak, unpubl. data).

In summary, the measurement of sex steroid levels represents one of the easiest and quickest methods of detecting impacts on the reproductive health of the fish. The level of impact on steroid levels required to elicit an irreversible biological response is

unknown, but an alteration in the production, regulation or circulation is indicative of changes in the reproductive system. MFOs can be used to indicate exposure to the effluent, but in situations where MFO activity levels are induced, steroid analyses are required as an estimate of biological response.

1.2 Theory

Although changes have been demonstrated in a number of gonadal sex steroids (testosterone, 11-ketotestosterone, 17β-estradiol and 17α20β-dihydroxy-4-pregnen-3-one), commercial assays are only available for testosterone and 17ß-estradiol. Measurements of the levels of these steroids will be indicative of impacts only if they are conducted at an appropriate season and stage of reproductive development, and if modifying factors are carefully standardized and controlled. The methods presented here have been widely used in endocrine research and have been used in recent years in pilot monitoring programs for impacts of toxicants on reproduction. Although the species may change, the steroid structures remain the same. These assays have been used successfully on a number of different species.

The production of gonadal sex steroids represents the end result of a complex chain of events that includes reception of external cues within the brain, integration of sensory information within the hypothalamic region of the brain, and the production of two regulatory neurohormones within the hypothalamus. These factors include gonadotropin releasing hormone (GnRH) which stimulates gonadotropin (GtH) production and release from the pituitary gland, and dopamine, which inhibits GtH production and release. Under the influence of the appropriate stimuli, GtH is secreted from the pituitary and acts on the testis or ovary to stimulate gonadal growth and the production of sex steroids. Normally, this system is controlled through a system of negative feedback, in which steroids feedback to the hypothalamus, the pituitary and the gonads to control their own circulating levels. In the case of fish exposed to bleached kraft pulp mill effluent, the normal

negative feedback mechanisms are impaired, resulting in depressed circulating steroid levels (Munkittrick *et al.* 1991b). Each step of the chain can be measured, with varying degrees of difficulty. The simplest is the measurement of circulating levels of the steroids in the blood, as described in this protocol. Since this is the last step in the chain, it has the potential to exhibit the most dramatic response.

1.3 Sampling requirements and modifying factors

The most important confounding biological variable is the reproductive state. For this protocol, fish undergoing active gonadal growth and development must be used. Only limited information is gathered from fish undergoing gonadal regression, which occurs immediately following spawning. Optimal sampling time is during the inter-spawning interval, or prior to spawning. Steroid levels (both 17β-estradiol and testosterone) reach peak concentrations in blood during the spawning season.

Fish population studies, as described in the Technical Guidance Manuals for Aquatic Environmental Effects Monitoring at Pulp and Paper Mills (Environment Canada 1991b) require that fish be captured in non-spawning (and non-migratory) seasons, for the purposes of assessing pulp mill impacts on fish populations. In order to detect reproductive changes associated with pulp mill exposure, fish must be collected at a time when they are actively synthesizing and utilizing steroids. In practical terms, this necessitates an interval of at least four months after spawning (in annually spawning species) to ensure that gonadal recrudescence and consequently circulating steroid levels have begun to increase after the gonadal resting stage. In male fish, testosterone is a dominant sex steroid. In females undergoing the early stages of ovarian growth (vitellogenesis), both testosterone and 17β-estradiol are present in the circulation. Signs of appropriate timing and elevation of blood steroids are indicated by an increased gonadal size over that found during the inter-spawning or resting period.

It should be emphasized that sampling near the times of the initiation of gonadal growth (and during final maturation for spawning), slight changes in reproductive timing between reference sites and exposed sites can cause statistically significant differences in circulating steroid levels. Site to site variation is not as sensitive to slight timing differences during the middle stages of gonadal development. It is crucial that all modifying factors be controlled as carefully as possible. Biological, environmental and procedural variables affecting steroid levels must be controlled when designing a monitoring program. Most field studies collect reference fish under similar conditions as the fish of interest so that confounding variables such as species, sex, maturity, size, migrations, time of day, water temperature, etc. is minimized. Using these designs in field studies, gonadal sex steroid levels can be very successful in detecting impacts on reproductive development.

Circadian variation has been reported in circulating steroid levels and the time of day in which the samples are obtained is a very important variable when sex steroid levels are to be measured. To minimize within-site variation, each sex must be sampled within a consistent 2 to 3 h time frame. This allows differences that may exist between sites to be detected more easily. It is essential that comparisons be made of fish at comparable stages of sexual development. This is particularly critical during the actual spawning period when sex steroid levels undergo rapid and dramatic changes. For example, comparisons can be readily made between sites by collecting samples at times of peak migration to the spawning regions.

It is critical that samples be collected in a similar manner at the reference and exposed sites. The capture technique, time to sampling, and sample handling must be controlled and similar between sites. Prolonged stress (caging fish) has been shown to affect circulating sex steroid levels (Van Der Kraak, unpubl. data). Consistency between sites will eliminate these potential problems.

2.0 SAMPLE COLLECTION AND STORAGE

2.1 Study design and choice of fish species

The Environmental Effects Monitoring study design requires that sampling include at least two species (at least one benthivore and probably one pelagic predator) (Environment Canada 1991b). Sample size will be based on the study design and requirements. A minimum of 6 fish of each sex are to be sampled for MFO analysis, and a comparable number of fish are to be sampled per sex per site for reproductive steroid levels, when they are measured. Under the requirements of the Environmental Effects Monitoring program (Environment Canada 1991a), blood sex steroid analyses are only recommended as a sensitive indication of reproductive impact. A prudent study design would involve collecting blood samples during fish sampling. Under the current conformation of the EEM program, these would only have to be forwarded for analysis if additional information was required on the potential for reproductive impact.

The selection of the reference or uncontaminated site is very important in a field study of this nature. It is preferable to collect fish at two reference locations if possible. In a river system, one immediately upstream of the source is ideal if movement of the fish into the contaminated area is restricted. In the lake situation, a site far enough away to prevent migration or in a lake in the same system. Sampling at two reference sites may not be required if a) fish are prevented by barriers from moving between the upstream and downstream sites, or b) sufficient data are available from other sources to validate the data from one reference site.

2.2 Field sampling procedures

There are a few alternatives for sampling. Blood samples can be collected by caudal puncture/severance, cardiac puncture or other appropriate method from live fish. If

caudal severance is used, the first few drops of blood must be avoided, preventing the collection of mucus from the cut. A sample of 1 to 3 mL of blood is sufficient to carry out the analyses. It is crucial that the blood samples be placed on ice as soon as collected. Blood samples can be collected in either heparinized or non-heparinized tubes and stored on ice until centrifugation. A maximum time delay of up to 8 h prior to centrifugation is allowable if the blood is kept cold. The handling of samples and analyses are identical whether plasma (heparinized sample tubes) or serum (non-heparinized) samples are to be collected. Centrifugation at 3000 rpm for a period of 10 minutes produces a supernatant which must be stored at -20°C until analysis. Multiple episodes of freezing and thawing must be avoided.

3.0 CHOICE OF STEROID ANALYTICAL METHODOLOGY

3.1 Radioimmunoassay

Several methods are available for measuring steroid levels in fish blood. The most practical of these and the techniques most commonly used at the present time is radioimmunoassay (RIA). Measurement of steroid hormones by RIA depends upon the availability of:

a) an antiserum specific to the steroid to be measured,
b) a radiolabelled form of the steroid,
c) pure unlabelled steroid for assay standards, and
d) a method by which antibody-bound steroid can be separated from free steroid.

For steroid RIA, a fixed concentration of radiolabelled steroid (b) is incubated with a constant dilution of specific antiserum (a) such that only 50% of the total steroid concentration will be bound by the antibody. When unlabelled steroid (c; or unknown samples) is added, there is a competition between radiolabelled steroid and unlabelled

steroid for the limited and constant number of binding sites on the antibody. As a result, the amount of radiolabelled steroid bound to the antibody will decrease as the concentration of unlabelled steroid increases. The concentration of bound radiolabelled steroid is inversely proportional to the amount of unlabelled steroid present in the standard or sample. The concentration of steroid present in blood samples is determined by comparison with the results obtained using graded doses of unlabelled steroids to generate a standard curve.

This technique has many advantages over other analytical procedures, including the availability of commercial reagents and kits, high sensitivity, and the capacity to analyze a large number of samples relatively inexpensively and quickly.

3.2 Alternate analytical techniques

Methods other than RIA are also suitable for measurement of steroids from fish blood. These include immunoradiometric methods in which the antibody is bound to a solid phase such as a polystyrene plate. Several variations of this method have been developed, including non-radioactive methods in which either fluorescent or enzyme-labelled hormone probes are utilized (e.g. fluorescent immunoassay [FIA]) or enzyme linked immunosorbant assay [ELISA]). However, these methods tend to be used exclusively in a research setting and are not as readily available as RIA on a commercial basis.

The use of high performance liquid chromatography (HPLC) is an alternate approach. This method has the advantage that several steroids can be measured simultaneously. However, this method is not particularly well suited to measurement of large numbers of samples, tends to lack the required sensitivity, and is very costly.

4.0 RIA PROTOCOL

There is no universal protocol for steroid hormone analysis by RIA. The choice of RIA buffer, incubation volumes, standard hormone concentrations and means of separating antibody-bound from free hormone vary from laboratory to laboratory. If researchers are using commercial RIA kits, all the necessary reagents and an established protocol will be provided. We outline an assay protocol that has been utilized for the past 12 years in one of our laboratories (Van Der Kraak *et al.* 1984; Van Der Kraak and Chang 1990; Van Der Kraak *et al.* 1990).

Commercial diagnostic kits for 17β-estradiol and testosterone measurements are available from a number of sources, including ICN Biomedicals Canada Ltd. (Mississauga, ON), Inter Medico (Markham, ON) and Amersham Canada Ltd. (Oakville, ON) (only examples are given, and are not to be construed as a complete list or as an endorsement or recommendation for use). Individual reagents can be purchased from a wide variety of sources. Radiolabelled steroids can be purchased from Amersham Canada Ltd. and New England Nuclear (Montreal, P.Q.). Unlabelled testosterone and 17β-estradiol can be purchased from Sigma Chemical (St. Louis, MO), Steroloids (Wilton, NJ) and Terrochem Laboratories Inc. (Mississauga, ON). Antisera can be obtained from ICN Biomedicals Canada Ltd., Radio Assay Systems Ltd. (Carson City, CA) and Sigma Chemical.

4.1 Equipment

The following materials and equipment are required:

a) refrigerator
b) refrigerated centrifuge capable of 3000 rpm, with adaptor for holding large numbers of 12 x 75 mm tubes
c) scintillation spectrophotometer
d) electronic balance (0.001 g sensitivity)

e) pH meter
f) fume hood
g) vortex mixer
h) stir plate with temperature regulation
i) pipettes or pipetting equipment with disposable tips (adjustable from 20 µL to 1000 µL)
j) repeater pipettes capable of dispensing 200 µL
k) repeater dispensers for ether and scintillation fluid (5 mL volumes)
l) disposable borosilicate glass test tubes (12 x 75 mm for RIA analysis and 16 x 150 mm for extraction)
m) scintillation vials
n) graduated glass cylinders (25, 100 and 1000 mL)

4.2 Sample preparation

Only a small proportion of steroid molecules in the blood exist in a free form. The vast majority of steroid molecules are bound by plasma proteins. If plasma was assayed directly for steroid content, plasma proteins would interfere with hormone measurement by competing with the antibody. To eliminate this problem, plasma samples are routinely mixed with organic solvents to separate steroids from the binding proteins. By simple extraction with diethyl ether, >95% of the steroid present in the plasma are recovered.

Ether is highly volatile and flammable. All steps outlined below involving ether must be completed in a fumehood.

1. Add known volumes of plasma from the samples to 16 x 150 mm borosilicate glass test tubes - eg. 200 µL, 100 µL or 50 µL (avoid repeat freeze-thawing of samples).

2. Adjust volume to 1.0 mL with distilled H_2O.

3. Add 5 mL of ether and vortex vigorously for 20 s.

4. Let stand for 10 min so that the ether and the aqueous phases separate (alternately, cover test tubes with parafilm and centrifuge at low speed for 2 to 3 min to separate the phases).

It is possible at this stage to 'snap-freeze' the samples by immersing the test tubes into a bath of dry ice and acetone. The aqueous component will freeze and the ether phase can be easily poured into the scintillation vial. If this approach is used proceed directly to step 7.

5. Carefully remove the upper organic phase and transfer to a glass scintillation vial.

6. Repeat steps 3, 4, and 5 and combine extracts to ensure that all steroid has been recovered from each sample.

7. Evaporate the ether phase in a warm (50°C) water bath under nitrogen.

8. Reconstitute sample with 1.0 mL of Phosgel (see below) and freeze (-20°C) until needed for the RIA.

Note: It is imperative to know the extraction efficiency of steroid from the sample. This must be examined by spiking samples with radiolabelled steroid (approximately 1000 cpm). Extraction efficiencies of >95% of added steroid are required. On a routine basis, it is often not necessary to correct for extraction efficiency once an effective and reproducible protocol has been developed.

4.3 Reagents and solutions

1. RIA Buffer

   -"Phosgel" (5.75 g Na_2HPO_4, 1.28 g $NaH_2PO_4 \cdot H_2O$, 1 g gelatin, 0.1 g Thimersol, and 800 mL distilled H_20)

   - heat at 45-50°C for 15 min to dissolve the gelatin and cool; adjust to pH 7.6 and fill to 1 L with distilled H_2O

2. Carbon Solution

   - 0.5 g activated carbon, 0.05 g Dextran T-70 (Pharmacia, Etobicoke, ON) and 100 mL Phosgel

3. Scintillation Cocktail

   - 2 L toluene, 1 L Triton X-100, 12 g 2,5-diphenyloxazole [PPO], 0.6 g 1,4-Bis[2-5-Phenyloxazolyl]Benzene [POPOP] or commercial scintillation cocktail such as Scintiverse (Fisher Sci., Toronto, ON)

4. Steroid Stock Solution

   - For 17ß-estradiol and testosterone, prepare stock solutions at 1000 ng mL^{-1} in absolute ethanol

5. Radiolabelled Steroids

   - 2,4,6,7-^{3}H-17β-estradiol and 1,2,6,7-^{3}H-testosterone (specific activity approximately 80 to 105 Ci $mmol^{-1}$)

Note:

a) Reagents 1 and 2 may be prepared and stored at 4°C for 2-3 days and 1 week respectively.

b) Reagent 3 must be stored in a dark, well-sealed bottle in a fume hood as toluene is highly flammable.

c) Reagents 4 and 5 can be stored at -20°C for up to 1 year.

4.4 General RIA format

The general format for the steroid RIA involves the following steps:

1. Pipette 200 µL of assay standard or unknown samples into 12 x 75 mm borosilicate glass test tubes.
2. Add 200 µL of radiolabelled steroid.
3. Add 200 µL of diluted antisera.
4. Incubate overnight at 4°C.
5. Add 200 µL of dextran-coated carbon solution and incubate for 10 min.
6. Centrifuge and decant supernatant into scintillation vials.
7. Add scintillation fluid and count.

As well, the RIA protocol requires that an estimate non-specific binding of radiolabelled hormone be conducted. This involves the same steps as outlined above, with the exception of step 3. For the determination of non-specific binding, 200 µL of assay buffer (Phosgel) is added in place of the specific antisera. The amount of radiolabelled hormone bound in the absenco of antisera is considered non-specific binding and correction is

made for this value in the assay calculations (see below).

4.5 Detailed RIA format

1. Hormone standards are prepared by adding 0.1 mL of stock solution to 24.9 mL of Phosgel. This represents the highest standard: 800 pg per 200 µL. A series of 1:1 dilutions are performed to prepare standards of 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 pg per 200 µL of Phosgel. The 0 hormone standard is prepared using Phosgel alone; this is also used for measurement of non-specific binding (see above). Standards should be prepared in triplicate.

2. Radiolabelled steroid (^{3}H) must be diluted with Phosgel to contain 5000 cpm per 200 µL.

3. Antisera dilutions are calculated so as to bind 40-55% of added ^{3}H-steroid in the absence of competitor.

4. Incubate at 4°C for 18-24 h (it is possible to decrease the time of incubation by conducting the assay at room temperature).

5. Tubes must be cooled to 0-4°C prior to the addition of the carbon solution by placing them in an ice/water bath.

6. The carbon solution must be cooled (as in 5) and placed on a magnetic stirrer. The carbon must be stirred when being added, or carbon will settle to the bottom of the beaker. Add 200 µL of the carbon suspension in order.

7. After the addition of carbon to the last tube, begin a 10 min incubation. During this period, vortex the samples and place the tubes in the centrifuge so that it can be

turned on 10 min after the addition of the carbon to the last tube.

Note: You must not add carbon to more tubes than can be held in the centrifuge at one time. If you have more samples than can be centrifuged in one run, include a second standard curve for those samples.

8. Centrifuge for 12 min at 3000 rpm at 4°C.

Note: The timing of 6,7 and 8 is critical. Once you commence the addition of carbon you cannot stop. Additionally you must add carbon to all tubes as quickly as possible so that the time interval between the first and the last tubes is minimized.

9. Carefully pour off the supernatant fluid into counting vials. Care must be taken to avoid shaking of the tubes and disturbing the carbon pellet.

10. Add 5 mL of scintillation fluid and measure radioactivity on a scintillation spectrophotometer.

5.0 DATA

5.1 Data calculation

Representative data for a standard curve for testosterone determination are shown in Table 1. From the example shown in Table 2, the testosterone content in the sample assayed was 75 pg $tube^{-1}$ as determined by interpolation. It is necessary to back-calculate to the concentration in the original sample of plasma extracted. In this case, the calculations would proceed as follows:

Volume of plasma extracted	100 µL
Reconstituted plasma volume	1000 µL
Volume Assayed in RIA	200 µL
Steroid Content	75 pg tube^{-1}

$$\text{Plasma content} = 75 \text{ pg} \times \frac{1000 \text{ µL}}{200 \text{ µL}} \times \frac{1000 \text{ µL}}{100 \text{ µL}} = 3750 \text{ pg mL}^{-1}$$

5.2 Additional data requirements

Steroid measurements are not made in isolation. Aside from associated environmental measurements described in other protocols (e.g. temperature, alkalinity, pH, etc), there are several measurements or sample collections that must be made on each fish sampled during the necropsy. These include: total body weight (g), gutted carcass weight (g - weight after removal of intestines and gonads), gonad weight (g), liver weight (g), fork length (cm), sex, and age. Age is determined by counting annuli in hard bony structures such as otoliths and cross-sections of fin ray sections, cleithrum or opercular bone, depending on the species. Use of scales is not recommended for fish greater than 5 y of age. From these measurements, the condition factor (CF = 100 x weight/ length^3), liversomatic index (LSI = liver weight/ total body weight - liver weight) and gonadosomatic index (GSI = gonad weight/ [total body weight - gonad weight]) can be calculated.

5.3 Statistics

Assuming that at least five fish have been caught at each site, the data are to be analyzed statistically following the study design. It is a general phenomenon that biological data are not distributed normally, but are skewed towards higher values. All data must be tested for normality and homogeneity of variance before applying statistical

Table 1. Typical data for testosterone assay standard curve.

Sample	Tube	Counts	Average Counts Bound	Net Counts Bound	Percent Reference Binding
Non-specific	1	105	117	-	-
	2	110			
	3	135			
0 pg	4	3032	3060	2943	100
	5	3097			
	6	3051			
3.125	7	2937	2903	2786	94.7
	8	2922			
	9	2850			
6.25	10	2710	2744	2627	89.3
	11	2800			
	12	2722			
12.5	13	2535	2514	2397	81.5
	14	2457			
	15	2549			
25	16	1998	2018	1901	64.6
	17	2013			
	18	2044			
50	19	1523	1480	1363	46.3
	20	1484			
	21	1433			
100	22	986	963	846	28.5
	23	945			
	24	957			
200	25	607	593	476	16.2
	26	602			
	27	571			
400	28	383	377	360	8.9
	29	380			
	30	369			
800	31	251	216	99	3.4
	32	200			
	33	198			

Table 2. Calculation of steroid content in unknown samples.

Sample	Tube	Counts	Average Counts Bound	Net Counts Bound	Percent Reference Binding[a]	Testosterone Content (pg tube^{-1})
1	1	1163	1147	1030	35.0	75
	2	1131				

[a] Net counts bound sample (1030) / Net counts bound 0 pg (2943)

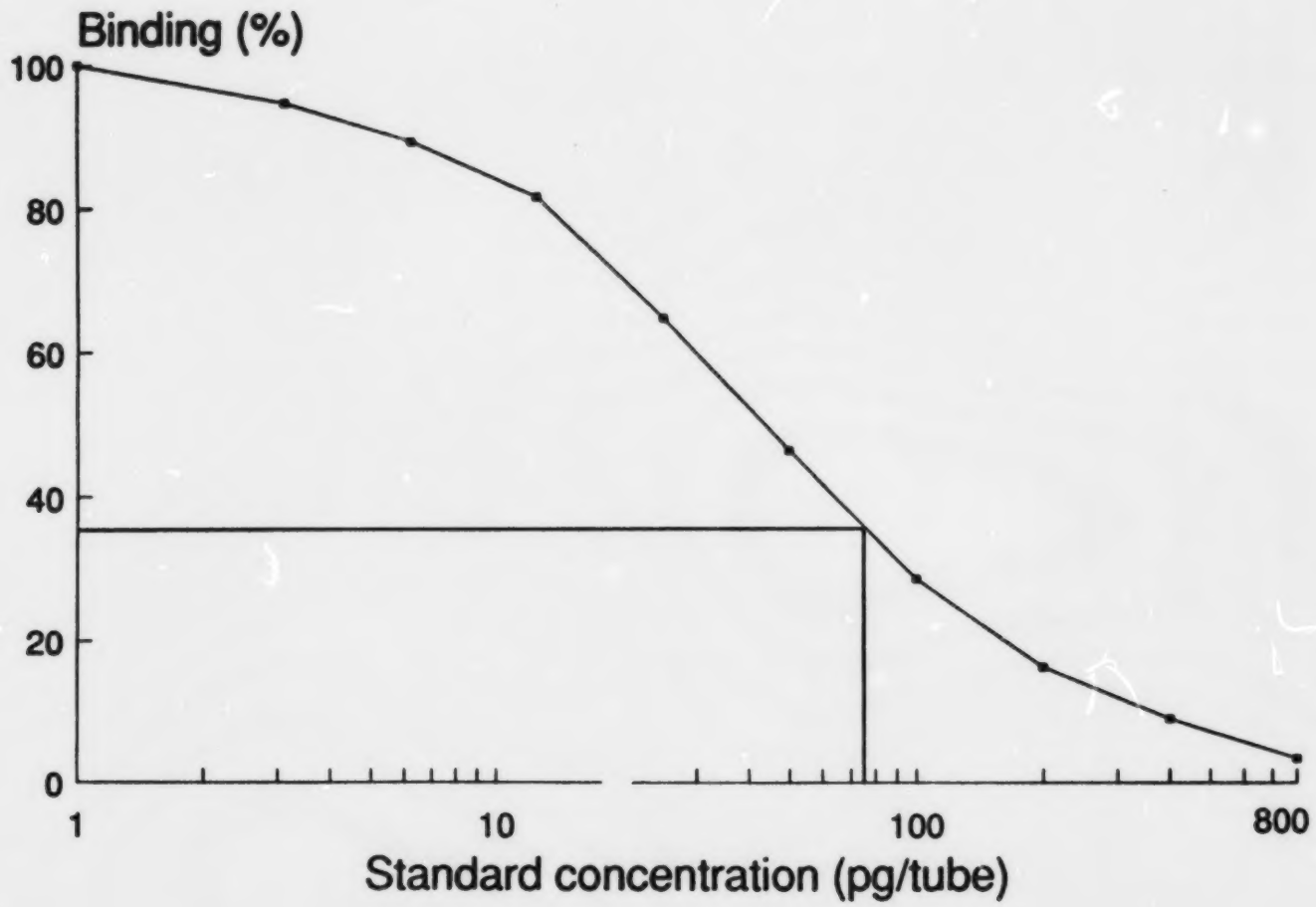

Figure 1. Sample standard curve for the data from Table 1.

tests appropriate for the study design. Data that fail these tests can often be normalized by a log transformation.

The appropriate analysis for comparing responses among sites where sex or some other variable may also have an effect, and where sample sizes are often unequal, is analysis of variance following the general linear model. Weight, length, and condition factor should not necessarily be correlated to steroid levels, but these relationships must be examined. Significant correlations may suggest the presence of confounding factors, such as habitat selection and exposure to the pulp mill effluent by different sizes of fish. The results should be interpreted accordingly and future study designs modified to eliminate bias.

Where analyses of variance have been applied, differences among sites can be tested with an appropriate multiple range test such as Tukey's. All differences and effects must be tested at a 95% probability level. If preliminary estimates of variance are available, the minimum sample size to detect a given difference (usually doubling of enzyme activity) and the probability of failing to detect a real difference (Type II error) should be calculated in advance as an aide to the study design. Relationships between steroid levels and the other measurements must be examined.

5.4 Definition of a response

There are no absolute or 'correct' values for steroid levels in fish. The best basis for judging the influence of an effluent is the pattern of responses among contaminated and reference sites. Assuming that sufficient fish (6-12) have been captured and analyzed at a minimum of one reference and two or more contaminated sites, a steroid impact would be significant if the following criteria are met:

a. Steroid levels are statistically significantly altered at contaminated sites relative to reference sites ($p<0.05$)

b. There is a recovery of steroid levels with distance downstream, suggestive of an 'exposure-response' relationship. A difference would be seen within a short distance downstream if there is dilution by a major tributary or the effluent enters an open lake. In a river, if there are no major tributaries, the minimum distance downstream between stations should be 30 km or below a major obstruction to fish migration.

c. The steroid impact is consistent between sexes of the same species, consistent among repeated surveys, or consistent among two or more species. Support for a steroid impact would also be given by changes to gonadosomatic indices that follow the same pattern of response as steroid levels.

5.5 Presentation

All raw data must be included in the final report. Data should be presented along with means, standard deviations, sample sizes, and results of all statistical tests of comparisons among sites and correlations among variables. Presentations of means and standard deviations in bar charts showing reference and contaminated sites is very helpful in understanding the results. The data must also be summarized in writing and any difficulties with sample collection or analyses, or any unusual observations or results, must be described and interpreted. Where other measurements have been made (e.g. temperature, chemical concentrations in sediments, etc), it is important to compare the measurements on fish, as a way of explaining possible causes of observed responses.

6.0 PERFORMANCE CHARACTERISTICS

There are several aspects to quality assurance and quality control (QA/QC) which must be incorporated into every report. These include the presence of blanks, replicates and

standards, and the repeatability of the assay. These blanks and standards are provided in the detailed protocol. There are also requirements for measurement of extraction efficiency (Section 4.2) and measurement of non-specific binding (Section 4.4) which must be included. Other aspects of QA/QC must be examined and reported in order to validate the steroid RIA, including parallelism, inter- and intra-assay variability, accuracy and upper and lower detection limits for the assay being used.

6.1 Parallelism

The most important facet of establishing assay validity is to determine whether compounds present in the sample interfere with the RIA. The most common approach to this potential problem is to analyze serial dilutions of the extract in the RIA. In this analysis, the dilutions must be parallel to the assay standard curve. In these analyses, it is not routinely necessary to assay dilutions of each sample. However, in the initial set-up and validation of the assay methodology, several plasma samples representing different sexes and reproductive conditions must be examined.

6.2 Accuracy

The accuracy of the RIA procedure must be established for each laboratory. This is accomplished by spiking plasma samples with known amounts of unlabelled steroid. Following RIA, comparisons are made of the quantities of added hormone with the measured concentrations. A regression analysis can be conducted and the slope of the regression line of hormone added versus hormone measured should be 1. The slope of this regression must be reported with the data. Statistical testing can be used to determine whether the value is significantly different from y=x (slope = 1.0). A statistically significant difference from 1.0 indicates the presence of interfering substances, and the assay protocol must be reexamined.

6.3 Precision

Measurements of both inter- and intra-assay variability are required to establish laboratory competence. It is critical that an aliquot from a common pool of plasma be analyzed in each assay. This sample should contain steroid at a concentration which will affect a 50% reduction in the binding of radiolabelled steroid. Multiple analyses of the same sample in subsequent RIAs permits the determination of inter-assay variability. This is calculated as the standard deviation divided by the mean for these determinations, and must not exceed 15%. This value must be reported with the data. Assays showing an extreme variance from the expected concentration in a common pool must be repeated.

In the same manner, the pool of sample must be measured repeatedly (> 5 times) in the same assay to allow determination of the intra-assay variability. This value must not exceed 10%, and must be reported with the data.

6.4 Assay detection limits

A common problem is in the establishment of the detection limit of the steroid RIA. There is no universal agreement as to the best means of determining this value. One method is to use two standard deviations of the binding achieved with the 0 hormone standard as the lower detection limit. Values below this range do not differ significantly from 0 hormone. Typically, these values are reported as being less than the lowest standard. Detection limits must be reported with the data.

The upper detection limit of the assay is represented by concentrations which give 10% total binding in the RIA. The upper detection limit does not have to be reported, but it is required that samples of very high steroid content (<10% of total binding in the RIA) be reanalysed after dilution to levels resulting in approximately 50% binding.

6.5 Specificity

Data on the cross-reactivity of the antiserum to related steroids must be calculated and reported. Typically, this involves comparing the ability of related steroid hormones to compete with the hormone in question (ie. 17β-estradiol or testosterone) for binding sites on the antibody. The specificity of the antiserum is calculated as the ratio of steroid concentration to the cross-reacting substance at 50% inhibition of maximum binding.

In general terms, an assay is considered to be highly specific if cross-reactivity to closely related compounds is <5%. However, consideration must be given to the presence, and concentration of the cross-reacting substance in the sample being analyzed. For example, a high amount of cross-reactivity would not be important if the likelihood of the cross-reacting substance being present in the sample was extremely low.

7.0 SUMMARY

Alterations in circulating levels of sex steroids have been found near bleached kraft pulp mills, and have been associated with a variety of other impacts. The current Environmental Effects Monitoring Requirements for Pulp and paper Mills recommends the measurement of gonadal sex steroids as a sensitive indication of the potential for reproductive impacts. Circulating levels can be measured with a variety of methods, but the only approach currently satisfying the requirements of being sensitive to pg levels and being widely commercially available is radioimmunoassay (RIA). There is no universal protocol for steroid hormone analysis by RIA, but this report outlines an accepted protocol which has been used successfully.

8.0 DISCLAIMER

Mention of trade names or commercial products constitute endorsement or recommendation for use by the Federal Government.

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